

Uremic concentrations of guanidino compounds inhibit neutrophil superoxide production

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Uremic concentrations of guanidino compounds inhibit neutrophil superoxide production.

Background. In uremia, diminished reactive oxygen intermediate (ROI) production is an important consequence of impaired neutrophil function. We have studied the effect of guanidino compounds, known uremic toxins, on neutrophil ROI production *in vitro*.

Methods. Neutrophils from healthy volunteers were exposed for three hours to individual or mixed guanidino compounds (GC_{mix}) at concentrations encountered in uremic plasma. After removal of guanidino compounds, neutrophils were activated by adhesion, N-formyl-methionyl-leucyl-phenylalanine (fMLP), phorbol 12-myristate 13-acetate (PMA), or opsonized zymosan, and superoxide production was measured by lucigenin chemiluminescence (CL). The direct effect of guanidino compounds on superoxide production in activated neutrophils was also measured. The energy status (ATP and creatine phosphate), antioxidant status (total glutathione), and glycolytic flux (lactate production) were measured.

Results. The GC_{mix} pretreatment decreased the superoxide production in activated neutrophils (fMLP or zymosan) by 50% ($P < 0.01$) and the ATP concentration by 60% ($P < 0.05$), and it inhibited glycolytic flux (lactate production) by 45% ($P < 0.01$), but did not alter glutathione concentration. Simultaneous exposure to GC_{mix} and activation did not inhibit nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity in cell lysates, but inhibited superoxide formation in zymosan-activated intact neutrophils, and this inhibition was reversed following removal of the guanidino compounds.

Conclusion. Guanidino-succinate, -propionate, and -butyrate were individually as potent as the GC_{mix} . Inhibition of neutrophil superoxide generation by guanidino compounds results from a depressed energy status. Uremic concentrations of guanidino compounds significantly inhibit neutrophil metabolism, and this has serious implications for their function in host defense.

Infection remains a major cause of death in patients with uremia [1–3], and the mortality rate of dialysis patients from infection is between 5 and 35% [2]. Neutrophil biology has consequently been widely studied in ure-

mia, and defects of phagocytosis, generation of reactive oxygen intermediates (ROIs), chemotaxis, adenosine 5'-triphosphate (ATP) generation, hexose monophosphate shunt activity, antioxidant enzyme activity [4–6], and accelerated apoptosis [7] have all been described. Although guanidino compounds are removed during dialysis, the concentrations remain significantly higher in hemodialysis and peritoneal dialysis patients than in healthy subjects [8].

We investigated ROI production by neutrophils, together with antioxidant and energy status, after exposure to a range of these compounds at concentrations that are pathophysiologically relevant in uremia.

METHODS

Neutrophil preparation

Neutrophils were prepared from healthy volunteers by the Histopaque-Lymphoprep double-layer method.

ROI monitored chemiluminescence

Light emission was characterized using selective enhancers of chemiluminescence (CL): 20 μ mol/L lucigenin for superoxide and 20 μ mol/L luminol for hydrogen peroxide and peroxynitrite. Luminol CL from hypochlorous acid was 95% quenched by using 5% fetal calf serum (FCS) throughout.

Adenosine 5'-triphosphate was measured by the luciferin-luciferase firefly luminescence method [9].

Total glutathione was measured as total intracellular glutathione (reduced glutathione [GSH] + oxidized glutathione [GSSG]) using the method of Tietze [10].

Lactate (glycolytic flux) was measured spectrophotometrically in extracts of cells plus media.

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase was measured by lucigenin CL in lysed activated neutrophils.

Pretreatment of neutrophils with guanidino compounds

Neutrophil suspensions (1×10^6 /mL) were incubated in Ca^{2+} / Mg^{2+} -free Hank's balanced salt solution (HBSS)

Key words: uremia, neutrophil reactive oxygen intermediates, bacterial infection, immune defense, host defense.

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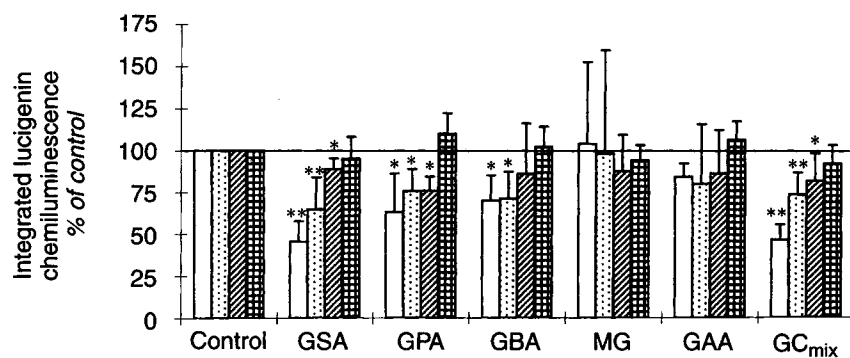


Fig. 1. Effect of pretreatment with a guanidino compound on neutrophil superoxide production. Total integrated counts over 60 minutes are shown in mean \pm SD. Symbols are: (□) zymosan; (▨) fMLP; (▤) adhesion; (▧) PMA; ** $P < 0.01$ and * $P < 0.05$ versus control. Abbreviations are: GSA, guanidinosuccinic acid; GPA, guanidinopropionic acid; GBA, guanidinobutyric acid; MG, methylguanidine; GAA, guanidinoacetic acid; GC_{mix}, mixed guanidino compounds; fMLP, N-formyl-Met-Leu-Phe; PMA, phorbol myristate.

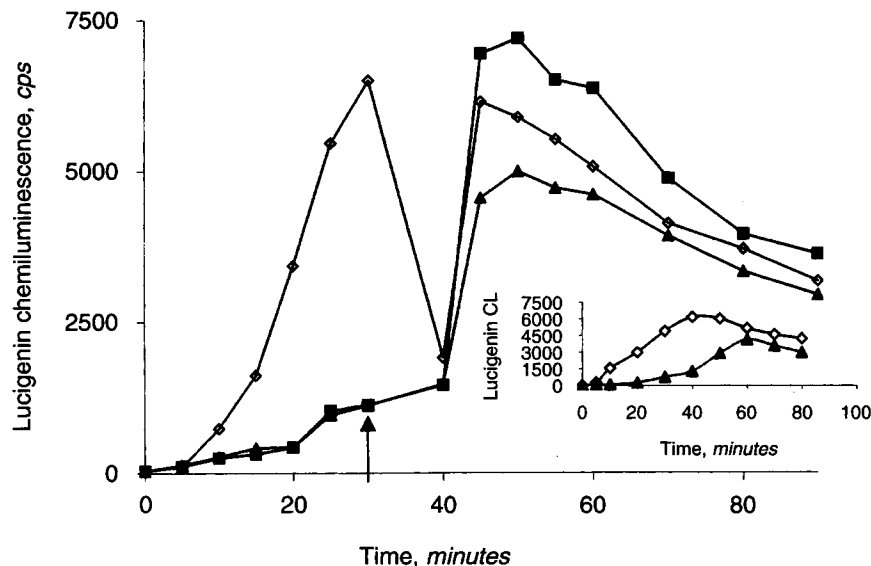


Fig. 2. Direct effect of guanidino compounds on superoxide production by zymosan-activated neutrophils. Neutrophils without (◇) and with (▲, ■) GC_{mix} were activated by zymosan. After 30 minutes of activation (arrow), the medium was replaced with Hank's balanced salt solution (HBBS; ◇, ■) or HBBS containing the GC_{mix} (▲). The insert shows an uninterrupted profile of chemiluminescence in neutrophils activated with zymosan in the presence (▲) or absence (◇) of GC_{mix}. Each value (counts/sec) is the average of 100 readings taken at each time point, and is representative of four separate experiments. In all cases the SD is $<2\%$.

containing 5% (FCS), in the presence or absence of guanidino compounds [guanidinosuccinate (GSA) 20 $\mu\text{mol/L}$, methylguanidine (MG) 10 $\mu\text{mol/L}$, guanidinopropionate (GPA) 5 $\mu\text{mol/L}$, guanidinobutyrate (GBA) 5 $\mu\text{mol/L}$, guanidinoacetate (GAA) 5 $\mu\text{mol/L}$, or a mixture of these (GC_{mix})] for three hours at 37°C. Immediately after treatment, cells were washed and then transferred into 35 mm culture grade dishes with lucigenin or luminol. The CL resulting from adherence to the dish and from neutrophil activation with opsonized zymosan A (40 $\mu\text{g/mL}$), phorbol 12-myristate 13-acetate (PMA; 20 nmol/L), or N-formyl-methionyl-leucyl-phenylalanine (fMLP; 20 ng/mL) was measured.

Direct effect of guanidino compounds on neutrophils

Untreated neutrophils were transferred to dishes containing guanidino compounds at the concentrations described, and lucigenin CL was monitored during adhesion and was followed by zymosan activation.

Effect of guanidino compounds on metabolic intermediates

Adenosine 5'-triphosphate, creatine phosphate, lactate, and glutathione were all measured as indices of

energy and antioxidant status after treatment with guanidino compounds for three hours.

RESULTS

The production of superoxide (lucigenin CL) following neutrophil adhesion was inhibited by preincubation of cells for three hours with GSA, GPA, and GC_{mix}, but was unaffected by GBA, MG, and GAA (Fig. 1).

Superoxide generation in neutrophils activated with zymosan or with fMLP and treated with a GC_{mix} was significantly inhibited compared with controls. GSA, GPA, and GBA alone each produced an inhibition that matched the GC_{mix}, whereas MG and GAA were without an effect (Fig. 1). However, when cells were activated with PMA, no inhibition with GC_{mix} occurred. Parallel inhibition of CL was observed when luminol was used in place of lucigenin (data not shown), indicating that the generation of peroxynitrite arising from the reaction of nitric oxide (NO) with O_2^- was not altered by the presence of guanidino compounds. In control neutrophils, lucigenin CL was abrogated by superoxide dismutase, and luminol CL was inhibited by 30% with 200 $\mu\text{mol/L}$ L-N-(1-iminoethyl)-ornithine, a nitric oxide synthase

(NOS) inhibitor. The number of cells adhering to dishes (DNA measurement) was unchanged by guanidino compound treatment (data not shown).

Direct effect of guanidino compounds on neutrophil superoxide production

The addition of a mixture of guanidino compounds at the time of neutrophil adhesion had no significant effect on the level of lucigenin CL ($83 \pm 15\%$ of control). However, there was a marked effect of guanidino compounds on lucigenin CL following zymosan activation (Fig. 2, insert). GC_{mix} caused a 40% inhibition of O_2^- production and a 30-minute delay in the start of CL emission. When the GC_{mix} was rinsed from the cells and replaced with fresh medium (Fig. 2, arrow), subsequent neutrophil O_2^- production was that of untreated neutrophils. However, if neutrophils were re-exposed to a GC_{mix} after rinsing, O_2^- production remained inhibited.

Nicotinamide adenine dinucleotide phosphate oxidase activity in resting and PMA-activated neutrophil lysates was unaltered by the addition of guanidino compounds even at a threefold higher concentration (data not shown).

Energy status (ATP, creatine phosphate, and lactate)

The steady-state (3 h) intracellular ATP concentration in resting neutrophils was significantly decreased (Fig. 3A) by GSA, GPA, GBA, and GC_{mix} ($P < 0.01$). Creatine phosphate concentrations were below the level of detection (<0.1 nmol/ 10^6 cells) in both control and treated neutrophils. The lactate production over three hours (intracellular and medium), a measure of glycolytic flux, was decreased by between 40 and 50% by GSA, GPA, GBA, and GC_{mix} ($P < 0.01$; Fig. 3B).

Intracellular glutathione in resting neutrophils was not significantly changed by treatment with individual or mixed guanidino compounds (Fig. 3C).

DISCUSSION

The formation of ROI by neutrophils is essential to maintain a defense against bacterial infection. This requires the initial formation of O_2^- by neutrophil NADPH oxidase and subsequent generation of the bactericidal agents hypochlorous acid and peroxynitrite. Our data demonstrate (1) that pretreatment of neutrophils with guanidino compounds inhibits production of superoxide by a metabolic effect (lowering of ATP and glycolytic flux), and (2) that the presence of guanidino compounds during neutrophil activation decreases the superoxide production during phagocytosis. This direct effect was not caused by either a diminution in the number of adherent cells or the inhibition of NADPH oxidase, nor to the quenching of lucigenin CL.

Differential changes in lucigenin and luminol CL measurements were used in combination to evaluate the rela-

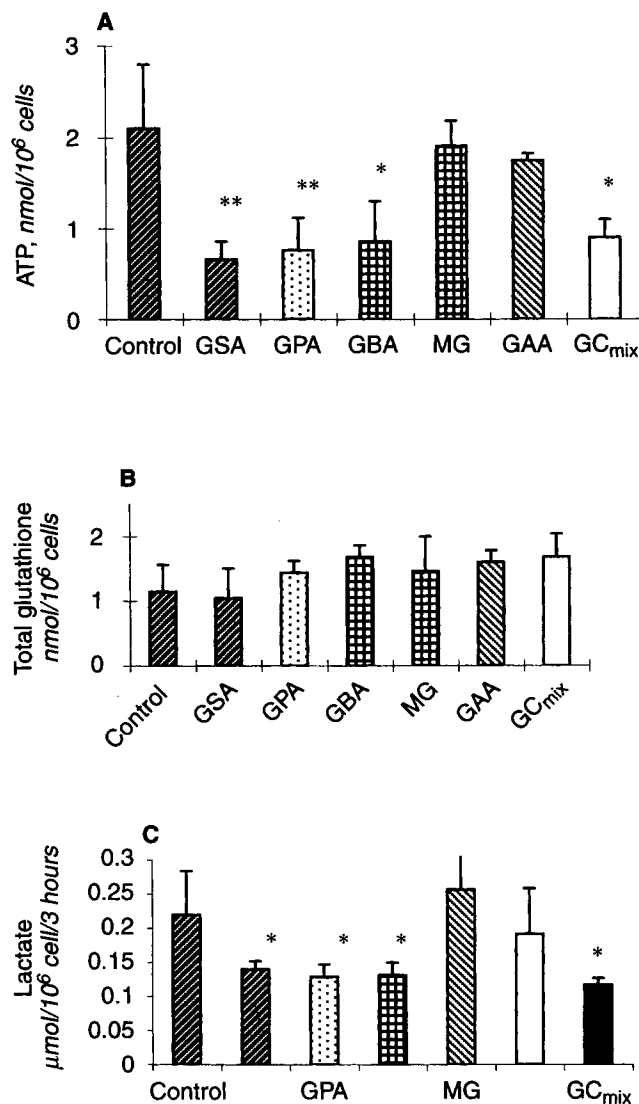


Fig. 3. Effect of guanidino compounds on resting neutrophil (A) ATP, (B) glutathione, and (C) lactate. Each value is the mean \pm SEM from 6 separate experiments. * $P < 0.05$ and ** $P < 0.01$ versus control. Abbreviations are in the legend to Figure 1.

tive contributions of O_2^- and nitric oxide (NO) during neutrophil activation. It has been suggested that some guanidino compounds can influence NO synthase activity [11, 12]. However, the pronounced inhibition of both lucigenin and luminol signals indicates that no change in NO production occurred during the treatment of neutrophils with guanidino compounds.

Neutrophil ATP is generated from glycolysis, and during neutrophil activation or phagocytosis, the ATP concentration quickly drops [13]. Our observed decrease in glycolytic flux will compromise the energy status, especially during cellular activation. There was a strong correlation between decreased ATP and O_2^- production ($r = 0.906$, $P < 0.005$). A relationship between energy status

and O_2^- production has been described [13], and we propose that this phenomenon explains the abnormal changes that we have observed.

As creatine phosphate pools are very low in neutrophils [13], reflecting the absence of mitochondrial-derived ATP, it is therefore unlikely that disruption in creatine phosphate metabolism is responsible for the depletion of energy stores. The addition of the guanidino compounds tested did not impose any oxidative stress on neutrophils since they were able to maintain a normal thiol status.

Guanidino compounds affected both phagocytic activity (adherence and zymosan) and G-protein-linked receptor-mediated events (fMLP). However, PMA, which acts via a cascade involving protein kinase C, was unaffected by guanidino compounds. Inhibition of neutrophil superoxide production by adenosine exhibits a similar differential effect between PMA and fMLP activation [14], and it is possible that accumulation of adenosine, a product of ATP breakdown raised during inhibition of glycolysis, is responsible for the effects reported. In addition, G protein coupling to fMLP receptor activation is heavily dependent on high-energy phosphate transfer from ATP to GDP, which in guanidino compound-treated cells will be impaired by the lowered energy status.

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